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Regulation of *EPC-1/PEDF* in Normal Human Fibroblasts Is Posttranscriptional

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Abstract The EPC-1 (early population doubling level cDNA-1) gene, also known as pigment epithelium-derived factor, encodes a protein belonging to the serine protease inhibitor (serpin) superfamily that has been reported to inhibit angiogenesis and proliferation of several cell types. We have previously reported that the EPC-1 mRNA and the secreted EPC-1 protein are expressed at levels more than 100-fold higher in early passage, G_0, WI-38 cells compared to either proliferating or senescent WI-38 fibroblasts. To examine the molecular mechanisms that regulate changes in EPC-1 gene expression in WI-38 cells, we isolated and characterized the human EPC-1 gene and determined the mRNA cap site. Transcriptional assays showed no change in the transcription rates of EPC-1 between young proliferating, quiescent, and senescent WI-38 cells. These results suggest posttranscriptional regulation of the EPC-1 gene. Reverse transcriptase polymerase chain reaction measurements (of hnRNA) indicate regulation at the hnRNA level. The regulation of the EPC-1 gene at the level of hnRNA can explain the observed slow increase in the steady-state EPC-1 mRNA levels when cells become quiescent. The reduction of EPC-1 mRNA levels that occurs when cells exit G_0 and are induced to proliferate can be accounted for by a reduction of the EPC-1 mRNA stability in stimulated cells as compared to quiescent cells. J. Cell. Biochem. 79:442–452, 2000.

Key words: EPC-1; human fibroblasts; PEDF

Normal human fibroblasts in culture have a limited proliferative capacity. After a defined number of replications, the cells gradually lose the ability to proliferate. There is some cell death, and eventually a population results that is viable but nonreplicative [Hayflick, 1965]. This process has been termed replicative senescence [Campisi, 1996; Cristofalo et al., 1998] and has provided an important model for studying the biology of aging. Details of the characteristics of the senescence process and the cellular changes that occur during senescence are reviewed in Cristofalo and Pignolo [1993]. In brief, there are changes in size, morphology, and gene expression, in the regulation and timing of the cell cycle, and in the turnover of the extracellular matrix.

In a previous study, we described differential expression of genes between replicating young and senescent fibroblasts [Doggett et al., 1992]. We named one of these differentially expressed genes *EPC-1* (early cell population doubling cDNA 1) [Pignolo et al., 1993]. The mRNA for this gene is expressed in young cells in G_0, achieved either through density-dependent inhibition of replication or by serum/growth factor deprivation. The expression of *EPC-1* mRNA and protein is 100-fold higher in these quiescent young cells than in either growing young cells or nongrowing senescent cells. The *EPC-1* gene codes for a 50-kDa secreted protein, which belongs to the general family of

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serine protease inhibitors and exhibits 30% identity to other members of this class (a degree of similarity that is typical of members of this gene family).

Human endometrial fibroblasts that produce *EPC-1* inhibit the growth of several endometrial carcinoma cell lines [Palmieri et al., 1999], and the same effect can be achieved in a dose-dependent manner using *EPC-1* protein produced in a baculovirus system [V.J.C., M.K.F. unpublished observations]. Addition of *EPC-1* protein also inhibits proliferation of glial cells [Sugita et al., 1997].

Essentially the same molecule has been described as a differentiation factor for retinal pigmented epithelial cells, and in this context has been named pigment epithelial cell–derived factor (PEDF) [Tombran-Tink et al., 1991]. The two molecules, PEDF and EPC-1, are identical except for a 2-bp polymorphism [Pignolo et al., 1993; Steele et al., 1993]. Several other polymorphisms located in exons 4 and 7 have also been reported [Slavc et al., 1997], and the gene structure for *EPC-1*/PEDF has been determined [Tombran-Tink et al., 1996; Singh et al., 1998]. The gene locus for *EPC-1*/PEDF has been mapped to 17p13.3, which is a site of frequent deletions in ovarian carcinoma lines [Phillips et al., 1993, 1996]. The *EPC-1*/PEDF protein has been shown to induce neuronal-like differentiation of Y79 retinoblastoma cells [Tombran-Tink et al., 1991] and to act as a neurotrophic factor for both retinal and cerebellar granule cells [Taniwaki et al., 1995]. A receptor for the *EPC-1*/PEDF protein has been identified on the surface of retinal pigmented epithelial and Y79 retinoblastoma cells, which may mediate the cellular responses to *EPC-1*/PEDF [Alberdi et al., 1999].

A secreted, 45-kDa serpin has been identified in a murine colon adenocarcinoma line [Kozaki et al., 1998]. This protein, named caspin, is the mouse homolog of *EPC-1*/PEDF. Secretion of caspin was inversely related to metastasis and the protein bound to the extracellular matrix, suggesting a causal role in metastasis. Recently, it has been shown that *EPC-1*/PEDF significantly inhibited endothelial cell migration with greater activity than thrombospondin, angiostatin, or endostatin [Dawson et al., 1999]. Furthermore, *EPC-1*/PEDF is capable of inhibiting basic fibroblast growth factor–induced neovascularization in vivo, suggesting a potential role for the protein as an inhibitor of angiogenesis [Dawson et al., 1999].

Quiescent human fibroblast cells express the *EPC-1* mRNA, and steady-state levels decrease on serum stimulation and entry into the cell cycle. This decrease may be regulated at the level of transcription, hnRNA processing, or mRNA stability. All three levels of regulation were examined to gain insight into the regulation of this quiescence-specific gene and the decreased level of expression during replicative senescence.

In this article, we report on the regulation of the expression of *EPC-1*/PEDF. The data presented here indicate that regulation in WI-38 fibroblasts is posttranscriptional and occurs at both the mRNA and hnRNA levels. Regulation at the hnRNA level may reflect mechanisms to regulate gradual accumulation of this protein in growing cells and thus the gradual attenuation of proliferation rate.

EXPERIMENTAL PROCEDURES

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. The cDNA plasmids used were as follows: human β-actin, pHFβ A-1, was a gift from Dr. R. Baserga (Thomas Jefferson University, Philadephia, PA); human MnSOD, pSOD-2, was a gift from Dr. S. Church (Washington University, St. Louis, MO); human ornithine decarboxylase, pODC10/2H, was a gift from Dr. N. J. Hickok (Lankenau Hospital, Philadephia, PA); and PEDF cDNA was a gift from J. Tombran-Tink (University of Southern California, Los Angeles, CA). The cDNAs used for Northern analyses were excised and purified before being used as probes. The cDNAs and genomic DNA fragments used in the nuclear run-on assays were linearized but not excised.

Cell Culture

Stock cultures of WI-38 fetal human diploid fibroblasts [Cristofalo, 1980] were grown in minimum essential medium, supplemented with basal medium Eagle vitamins, 2 mM L-glutamine and 10% fetal bovine serum (FBS). Cultures were considered to be at the end of their replicative lifespans when they were unable to complete one population doubling during a 4-week period that included 3 consecutive weeks of refeeding with fresh medium containing 10% FBS. WI-38 cells at early passage

(young) were seeded at $3 \times 10^3/cm^2$ and grown for 3 days. This protocol was chosen to ensure that the cells were subconfluent and therefore possible effects of density on growth due to contact inhibition were minimal. Subsequently, the cells were serum deprived for 3 days in MCDB-104 medium to ensure G_0 arrest. At this point, the cells were stimulated for various times with FBS to a final concentration of 10% (vol/vol). For determination of the *EPC-1* mRNA half-life, the cells were treated with the following RNA synthesis inhibitors: α-amanitin (7 μg/ml) or 5,6 dichloro-1-β-ribofuranosyl-benzimidazole (DRB) (30 μg/ml).

RNA Isolation and Northern Analysis

Total RNA was isolated by the acid guanidinium thiocyanate/phenol-chloroform method [Chomczynski and Sacchi, 1987] modified to remove contaminating carbohydrates [Puissant and Houdebine, 1990]. Northern analysis was performed as previously described [Keogh et al., 1996]. Briefly, 10-μg aliquots of each sample were glyoxylated and fractionated in 1.2% agarose gels. Gels were then treated with 50 mM NaOH for 30 min, neutralized with 100 mM Tris pH 7.5 for 30 min, and RNA was transferred to Nytran Plus nylon membranes by electroblotting in 1X TAE (40 mM Tris-acetate, 1 mM EDTA), pH 7.8. After transfer, the membranes were washed for 10 min in 2X SSPE, air dried, and the RNA was UV cross-linked to the membrane. Blots were hybridized under standard conditions using a [^{32}P]-labeled full-length *EPC-1* cDNA. As controls for loading, the membranes were probed using a [^{32}P]-labeled, full-length human β-actin cDNA, and a [^{32}P]-labeled full-length cDNA of human ornithine decarboxylase (ODC). All probes were radiolabeled using the High-Prime random-primer labeling reagent, according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN). After hybridization, the blots were washed three times 15 min in 0.1X SSC, 0.5% sodium dodecyl sulfate at 45°C, and exposed to Kodak XAR-5 film (Kodak, Rochester, NY) or BioMax film at −70°C. Signal intensities were quantified using a Hewlett Packard Scanjet II scanner with ImagequaNT software from Molecular Dynamics (Sunnyvale, CA).

Primer Extension Analysis

Nine *EPC-1* genomic clones were isolated from a WI-38 fibroblast-derived genomic DNA library (Stratagene, La Jolla, CA; cat. no. 946204). Three of the clones were further characterized by restriction endonuclease analysis and partial sequencing (Genbank accession numbers U57444–57450). The *EPC-1* transcription start site was determined by primer extension, as described by Boorstein and Craig [1989], using an antisense primer complimentary to the PEDF cDNA bp 165–139, (5′-TGTGCCCGAGGAGGGCTCCAATGCAG-3′), using 50 μg of WI-38 RNA or 50 μg of yeast tRNA as a negative control and 2 ng in vitro transcribed PEDF RNA as a positive control.

EPC-1 Promoter Assays

A 711-bp genomic fragment overlapping the initiation site and extending into the promoter region of *EPC-1* (bp −680 to bp +31) was ligated in the sense orientation into the Xho I and Hind III sites of the pGL2- basic plasmid, a promoterless luciferase vector. The plasmid was cotranfected into early-passage WI-38 cells (seeded at 4×10^4) together with the LNCZ expression vector, which contains the β-galactosidase cDNA under control of the CMV promoter. Transient transfections were carried out according to the manufacturer's protocol using the cationic lipid reagent LipofectAMINE Plus, along with reagents that improve transfection efficiency that were provided by Life Technologies. As a negative control, sister cultures were cotransfected with the pGL2-basic plasmid together with the LNCZ expression vector. β-Galactosidase activity was used to estimate transfection efficiency. In repeated assays, the transfection efficiency was approximately 2–5% as judged by positive staining for β-galactosidase. Transfected cultures were lysed in luciferase lysis buffer (Promega, Madison, WI) and luciferase activity was determined according to the manufacturer's instructions using a Turner TD-20e luminometer. The relative promoter activities were normalized by dividing the result of the luciferase assay by that of the β-galactosidase assay in each sample.

The genomic sequence of the EPC-1 gene has been entered into the geneBank database under accession numbers U57444–U57450.

Nuclear Run-on Analysis

Isolation of intact nuclei from WI-38 cells and nuclear run-on assays were performed as

described by Allen et al. [1995] with the exception that 2×10^6 cpm/ml of labeled nuclear RNA was used for the hybridizations.

Reverse Transcription-Polymerase Chain Reaction

Total RNA to be analyzed by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was treated with DNAse I to remove contaminating genomic DNA. Briefly, 50 μg of each RNA sample, was incubated with 10 U DNAse I in a solution containing 20 mM Tris-Cl pH 8.4, 2 mM $MgCl_2$, 50 mM KCl, and 10 U RNAsin, at 37°C, for 30 min. Reactions were terminated by the addition of EDTA to a final concentration of 2 mM and incubation at 65°C for 10 min. The DNAse I-treated RNA was subsequently purified by phenol-chloroform extraction and ethanol precipitation. Two microgram of RNA from each sample was reverse transcribed in a total volume of 20 μl, using 2 μM primer and 400 U MMLV reverse transcriptase (Gibco BRL) in a reaction solution containing 50 mM Tris pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 10 U RNasin, 0.1 μg/μl bovine serum albumin and 20 μM dNTPs. Primers were used to reverse transcribe the *EPC-1* premessenger RNA that recognize the first (5′-CTCATCCACTCACCCTTTTG-3′), third (5′- CTCAGCCACGTTTATGCAGA-3′) and sixth (5′-CTGTCGGATCTCAAAGGTCA-3′) intron of the gene. The primer (5′-TGTCTGGGCTGCTGATCA-3′), which was derived from a sequence located in the fourth exon of *EPC-1* was used to reverse transcribe *EPC-1* mRNA and the primer (5′-ACCTCCATGATGCTGCTTAC-3′) was used to reverse transcribe β_2-microglobulin mRNA to be used for standardization. Two microliters from each reverse transcribed cDNA sample were amplified. The various primer sets used in the PCR reaction are as follows: exon 1 and intron 1 (5′-CTCATCCACTCACCCTTTTG-3′; 5′-CTCATCCACTCACCCTTTTG-3′); exon 3 and intron 3 (5′-CTCAGCCACGTTTATGCAGA-3′; 5′-CTCAGCCACGTTTATGCAGA-3′); and exon 6 and intron 6 (5′-CTGTCGGATCTCAAAGGTCA-3′; 5′-CTGTCGGATCTCAAAGGTCA-3′). The primer set (5′-TGTGCAGGCTTAGAGGGACT-3′) and (5′-TGTCTGGGCTGCTGATCA-3′′ derived from sequences located in exon 4 and exon 1, respectively, were used to amplify a 412-bp fragment of the *EPC-1* mRNA. The primers (5′-TCACGTCATCCAGCAGAGAA-3′) and (5′-ACCTCCATGATGCTGCTTAC-3′) were derived from sequences of the β_2-microglobulin gene and were used to amplify a 286-bp fragment of β_2-microglobulin. All PCR reactions were performed using the following protocol: one cycle of 95°C for 5 min, 55°C for 4 min and 72°C for 4 min, followed by cycles of 92°C, 55°C, and 72°C, for 30 s each step. The exact number of cycles for each sample is indicated in the Figure 1 legend.

The amplified fragments were separated on 2% agarose gels and transferred onto 0.2-μm Nytran Plus nylon membranes. Each blot was probed using a [γ^{32}P] end-labeled oligonucleotide probe. The probes used recognized sequences located within the amplified fragments and were derived from exon 1 (5′-ATACGCTGCAGCTCCACACCCAGCCTA-3′), from exon 3 (5′-CTCCTGTCTCCTCTCAGTGT-3′) and from exon 6 (5′-CTGAATCCAAGCCATA-3′) of the *EPC-1* gene. For the β_2-microglobulin reactions, a probe (5′-GTGGAGCATTCAGACTTGTC-3′) that recognizes the β_2-microglobulin cDNA was used. After hybridization, the membranes were exposed to Kodak XAR-5 film at −70°C.

RESULTS

Nuclear Run-on Analysis of the EPC-1 Gene

Nuclear run-on analysis of *EPC-1* was performed using nuclei from early passage fibroblasts in five different growth states as well as from senescent fibroblasts. The selections of the growth states were based on the relative steady-state expression of the *EPC-1* mRNA. We selected conditions in which the *EPC-1* mRNA was absent by Northern analysis, as well as conditions in which the mRNA levels were accumulating or in which mRNA was present at high levels. In none of these conditions was any significant difference seen in the relative rate of transcription of the *EPC-1* gene when normalized to genomic DNA. A lower incorporation and overall signal strength was always obtained from the senescent nuclei, most likely because of an overall decrease in the rate of RNA synthesis in these cells [Phillips et al., 1990].

As a positive control for the nuclear run-on technique, transcription of the c-fos locus was measured after 30 min of serum stimulation (Fig. 1B). In accordance with published results [Greenberg and Ziff, 1984], transcription of c-fos was strongly induced by the addition of

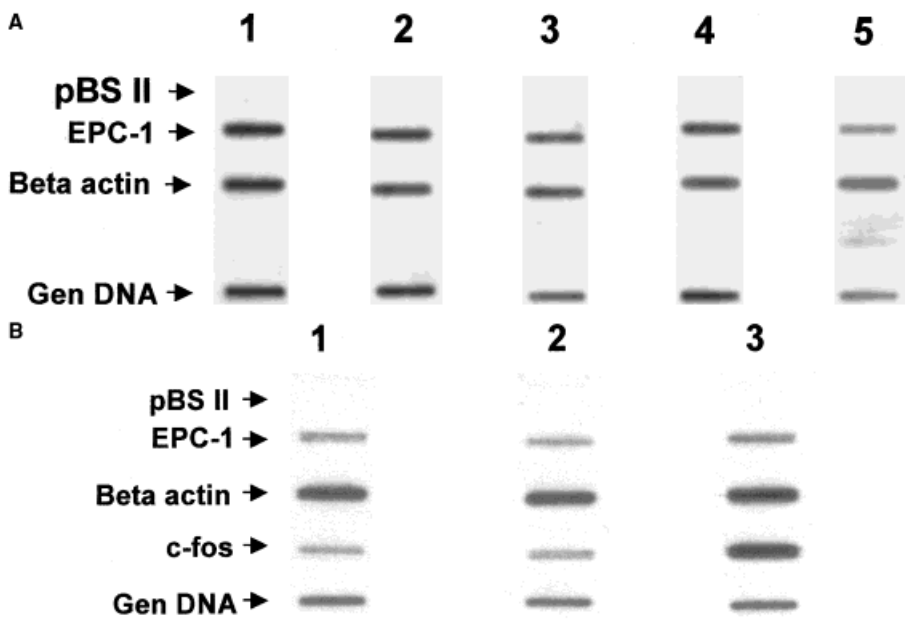

Fig. 1. Nuclear run-on analysis of the *EPC-1* transcription rate. Nuclear run-on assays were performed using nuclei isolated from 1) early passage, replicating; 2) early passage, subconfluent serum-deprived quiescent; 3) early passage, entering quiescence; 4), early passage, confluent quiescent; or 5) senescent cells (**A**). Transcription was then allowed to proceed in vitro. After extraction, ^{32}P-labeled nuclear RNA was hybridized to slot blots containing 10 μg of linearized DNA. Slots contain: pBluescript II (pBSII), *EPC-1*/PEDF cDNA (EPC-1), β-actin cDNA (β-actin), or 15-ng genomic DNA (gen DNA). **B:** Run-on assays were performed using nuclei isolated from cultures under the following conditions: 1) early passage, replicating; 2) early passage, subconfluent serum deprived, quiescent; 3) early-passage serum deprived for 48 h followed by 30-min serum stimulation. Slots contain: pBluescript II (pBSII), EPC-1/PEDF cDNA (EPC-1), β-actin cDNA (β-actin), c-fos genomic DNA exon 1 through exon 4 (c-fos), or 15 ng genomic DNA (gen DNA).

serum to serum-starved cells There was a low-level signal for c-fos expression in quiescent cells, most likely because of our use of a large genomic clone as a probe. This probe contained some repetitive elements, yet the relative difference observed between serum-starved and serum-stimulated cells was similar to that in the original report by Greenberg and Ziff. As other controls for the nuclear run-on assays, experiments were performed using single-strand probes derived from the 5′ and 3′ portions of the *EPC-1* cDNA. Similar control experiments were performed in the presence of 0.6% sarkosyl (data not shown). In no case did we observe any significant change in the transcription rate of the *EPC-1* gene.

EPC-1 Promoter

To examine the activity of the *EPC-1* promoter, we isolated and characterized three *EPC-1* genomic clones. We determined the cap site of the *EPC-1* mRNA using primer extension (Fig. 2). In WI-38 fibroblasts, the transcription initiation site was 62 bp 5′ of the ATG in the *EPC-1* cDNA, at the sequence, CCTC**A***GTGT. This sequence was previously identified as a weak transcriptional initiator element, very similar to the initiator sequence of the human terminal deoxytransferase gene [Javahery et al., 1994; Martinez et al., 1994]. The same site was identified in three primer extension experiments. It is important to note that the identical initiation site was also obtained using S_1 nuclease analysis (data not shown). The initiation site for *EPC-1* identified in pigmented retinal epithelial cells is 74 bp 5′ to the initiation site identified in this study [Tombran-Tink et al., 1996]. The initiation site for the *EPC-1* mRNA was only visible in the primer extension analysis when RNA derived from early-passage, serum-deprived cultures was used (see lane 2 of Fig. 2). This was consistent with the results of Northern blot analyses in that the mRNA for *EPC-1* was only detectable in RNA samples derived from early-passage, quiescent cultures.

Sequence analysis of the PEDF/EPC-1 promoter indicates that the 2,000-bp region 5′ to

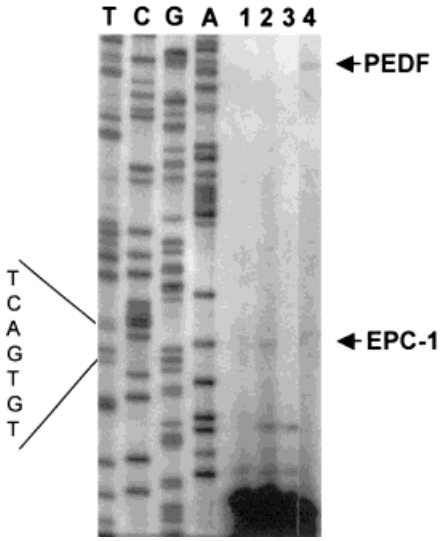

Fig. 2. Primer extension analysis of the *EPC-1* cap site. Fifty micrograms total RNA was used in each primer extension reaction performed as described by Boorstein and Craig using the 5′-TGTGCCCGAGGAGGG CTCCAATGCAG-3′ primer. Lanes 1–4 contain a sequencing reaction obtained using the same primer as used in the primer extension experiment and a cloned PEDF cDNA. The experimental samples were derived from cells that were early-passage proliferating (lane 1), early-passage quiescent (lane 2), senescent (lane 3), and yeast tRNA + 2 ng in vitro transcribed PEDF RNA as a control (lane 4). The cap sites for PEDF and EPC-1 are marked with arrows on the right side of the figure.

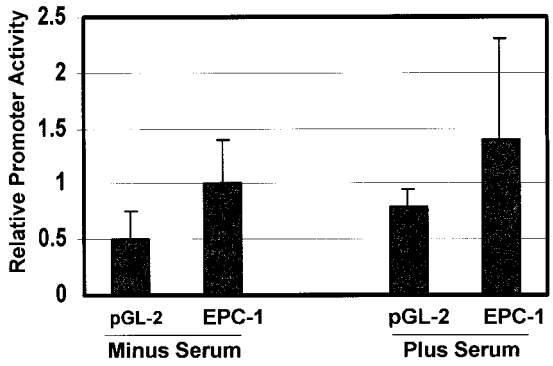

Fig. 3. Relative *EPC-1* promoter activity in quiescent versus serum-stimulated cultures. WI-38 cells were transfected with either pGL2-basic, a control plasmid lacking any promoter elements, or an *EPC-1* promoter/luciferase construct in the same pGL2 vector. These plasmids were cotransfected with a plasmid containing the β-galactosidase cDNA under the control of a CMV promoter. After transfection, cells were placed into serum-free medium (MCDB-104) or MCDB medium containing 10% serum for 48 h. The cells were then harvested, and luciferase activity of the cell lysates was determined. The data were normalized for transfection efficiency using β-galactosidase activity from the same lysates (N = 3). The experiment presented here is representative of several promoter studies using both chloramphenicol acetyltransferase and luciferase reporter genes.

the initiation site is unusually rich in ALU sequences and lacks many of the common promoter elements, such as TATA and CCAAT boxes or a GGGCGG element. To analyze that activity of the promoter, we limited our studies to a 711-bp promoter fragment (−680 to +31) to exclude these ALU sequences. The activity of this *EPC-1* promoter fragment was slightly higher in serum-stimulated cells compared to serum-deprived cells (Fig. 3), but the difference was not significant by standard statistical analysis (Student's *t*-test). Furthermore, the activity of this *EPC-1* promoter fragment is very weak. In fact, the *EPC-1* promoter is weaker than the SV40 early promoter (data not shown), which is itself a relatively weak promoter [Zenke et al., 1986]. This result, as well as the extensive nuclear run-on data, suggest that transcriptional control does not play a major role in regulation of the *EPC-1* gene under the conditions tested.

Changes in *EPC-1* mRNA Stability

We next sought to examine changes in *EPC-1* mRNA stability. Serum-starved, low-density, WI-38 fibroblast cultures were stimulated with media containing fetal bovine serum, and *EPC-1* mRNA levels were measured by Northern blot analysis. Examination of the kinetics of the gradual decrease in *EPC-1* mRNA levels reveals a 50% reduction in *EPC-1* mRNA levels, relative to time 0, by 15 h after stimulation. It should be noted that *EPC-1* mRNA levels were elevated initially after serum stimulation (Fig. 4). This phenomenon is not unique to *EPC-1*. For example, the mRNA levels of the cyclin-dependent kinase inhibitor p21 and the growth-arrest-specific (gas) genes showed a similar transient increase after serum stimulation [Ciccarelli et al., 1990; Del Sal et al., 1992]. This initial transient increase in *EPC-1* mRNA levels causes the rate of decline to be underestimated using the 0 time point as a reference. However, the zero time point was still used as a baseline for *EPC-1* mRNA levels to provide a conservative estimate of the rate at which *EPC-1* mRNA levels decline after serum stimulation. At 36 h, the levels of *EPC-1* were undetectable by Northern blot.

To determine whether there was a change in the half-life of the *EPC-1* mRNA that contributes to the decline in mRNA levels after serum

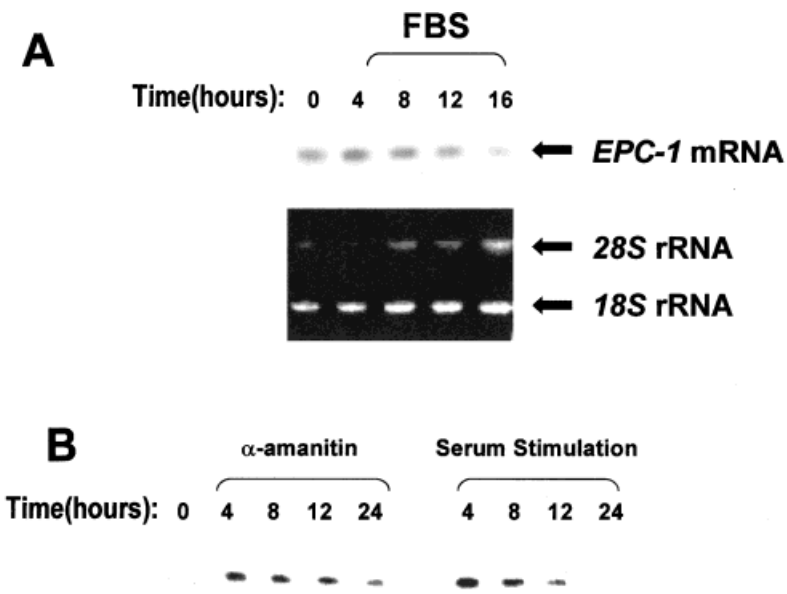

Fig. 4. **A:** Northern analysis of the *EPC-1* mRNA decay rate in response to serum. Cultures of WI-38 cells were seeded and grown for 3 days. The cells were then placed into serum-free MCDB-104 for 72 h. These cells were then serum stimulated. In this condition, *EPC-1* mRNA levels are elevated (time 0). RNA was isolated during 16 h after serum stimulation (4, 8, 12, 16 h) and subjected to northern analysis for *EPC-1* mRNA levels. Ethidium bromide staining of the agarose showing 18S and 28S RNA bands is shown below the *EPC-1* Northern blot. **B:** Northern blot directly compares the level of EPC-1 mRNA between serum deprived cells that were treated with α-amanitin and serum-deprived cells that were stimulated with serum-containing medium. This allows a direct comparison of the half-life of the *EPC-1* mRNA with its decline after serum stimulation. A calculation of the time that is required for a 50% reduction in *EPC-1* mRNA levels under these conditions is contained in Table I.

stimulation, the half-life of the *EPC-1* mRNA in quiescent cultures was determined and compared to the kinetics of the disappearance of the mRNA after serum stimulation. Two different inhibitors of RNA synthesis, α-amanitin and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), were used (Table I), whereas ornithine decarboxylase (ODC) and β-actin, with published half-lives of 8 and 20 h, respectively [Hsieh and Verma, 1988; Guttridge et al., 1993], were used for internal reference. The half-life of *EPC-1* was 25 and 29 h in the presence of α-amanitin or DRB, respectively. The β-actin half-life was 19 and 19.5 h, respectively. The half-life of ODC in the presence of DRB in our experiments was 8 h.

From the above analysis, we concluded that the half-life of the *EPC-1* mRNA, as measured in quiescent cultures, was much longer than the time required to achieve a 50% reduction in mRNA after serum stimulation. Clearly, the stability of *EPC-1* mRNA must be decreased after serum stimulation. Experiments to measure the half-life of *EPC-1* mRNA after serum stimulation showed that addition of either

TABLE I. Stability of EPC-1 mRNA: mRNA Half-Lives in Serum-Free Medium Versus Decline After Serum Stimulation[a]

Inhibitor	EPC-1 (h)	β-actin (h)	ODC
α-Amanitin	25	19	ND
DRB	29	19.5	8
Serum stimulation	12	NA	NA

[a]Subconfluent cultures of WI-38 cells were placed into serum free medium for 72 h to induce expression of *EPC-1* mRNA. The cultures were then treated with either of three RNA synthesis inhibitors, α-amanitin or 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB). Total RNA was isolated at 4-h intervals after addition of RNA synthesis inhibitors for up to 52 h. Levels of *EPC-1* mRNA were determined by Northern blot analysis and normalized to 28S RNA. The half life of *EPC-1,* β-actin, and ornithine decarboxylase (ODC) were determined using the equation: $\mathrm{Ln}([\mathrm{mRNA}]_t/[\mathrm{mRNA}]_{t0}) = -k_{obs}t$. Below the half-life of *EPC-1* is an estimation of the time that is required for a 50% decline in *EPC-1* mRNA levels as measured by Northern blot analysis after serum stimulation of quiescent, serum-deprived WI-38 cells at early passage. Because the mRNA level of *EPC-1* is constant in serum-deprived cultures, this measurement is not included. In addition, because β-actin and ODC increase in expression after serum stimulation, no numbers are given for these mRNAs.

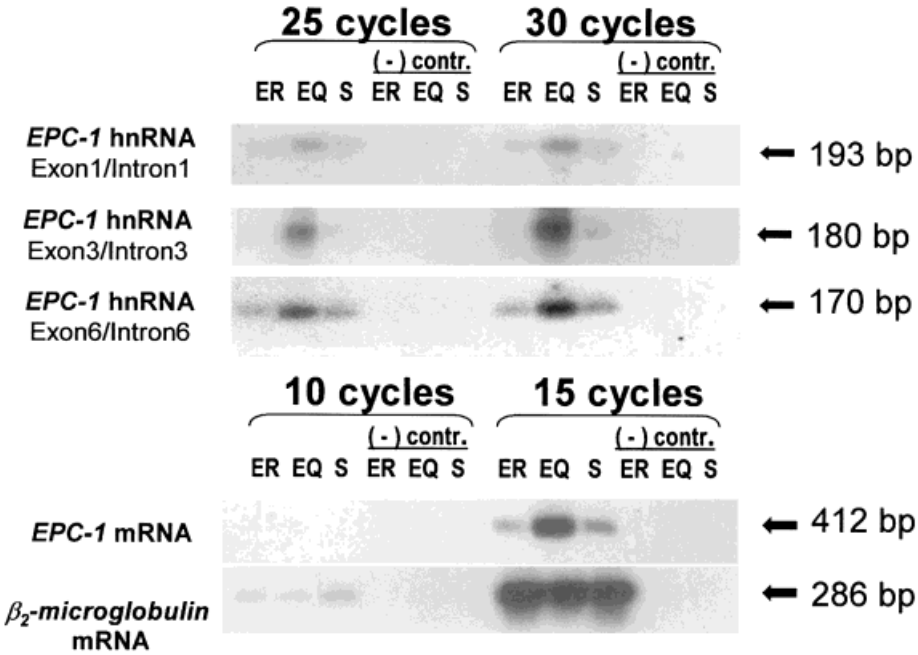

Fig. 5. The steady-state *EPC-1* hnRNA levels in proliferating, quiescent, and senescent cells. RNAs from early passage replicating (ER), early-passage quiescent (EQ), and senescent cells (S) were reverse transcribed using oligonucleotides (see Materials and Methods) derived from sequences in the first, third, or sixth introns of *EPC-1*. Reactions without reverse transcriptase provide negative controls and are labeled (−) contr. The *EPC-1* mRNA levels were measured in each sample to ensure that the RT-PCR measurements were reflective of the RNA levels as indicated in Northern blot experiments. Note that the signal for the mRNA is visible after 15 cycles of amplification, whereas the hnRNA is visible only at 25–30 cycles, reflective of the relative difference in abundance. As a control for mRNA loading, levels of β_2-microglobulin mRNA were measured in the same samples.The amplified cDNAs were subjected to Southern blot analysis using oligonucleotides derived from sequences in the first, third, or sixth exons as probes. The probes did not overlap the primers used in the RT-PCR, and the expected sizes of the PCR products are indicated in each case.

α-amanitin or DRB resulted in a 25-29-h half-life for *EPC-1* mRNA in serum-stimulated cultures. This half-life was similar to that obtained in serum-deprived cells (Table I) and was much longer than the 15 h required for a 50% reduction in *EPC-1* mRNA levels in the absence of the RNA synthesis inhibitors. It is possible that inhibition of RNA synthesis inhibits the synthesis of a protein required for degradation of the *EPC-1* mRNA, thus stabilizing the message. The addition of either of the protein synthesis inhibitors, puromycin or cycloheximide, also stabilized the *EPC-1* mRNA after serum stimulation (data not shown), further indicating a role for a labile protein. From the combined results of these experiments, we conclude that the stability of the *EPC-1* mRNA is reduced after serum stimulation.

Alterations in *EPC-1* hnRNA Levels Parallel Changes in mRNA Levels

Steady-state levels of *EPC-1* hnRNA were examined by RT-PCR using primers spanning intron–exon boundaries of the *EPC-1* gene. Aliquots of the RT-PCR reactions were taken at intervals during the PCR reaction. The reaction products were then subjected to Southern blot analysis using a probe distinct from the primers used for PCR. This produces a very sensitive measurement of the PCR products and allows results to be analyzed after a relatively low number of PCR cycles. This is critical to ensure that the reaction is measured during the linear phase while none of the components are limiting.

When the *EPC-1* hnRNA levels were examined, the levels of hnRNA mirrored those of *EPC-1* mRNA (Fig. 5). In quiescent cultures, high levels of hnRNA were found, whereas the levels in proliferating or senescent cultures were dramatically lower. Similar results were obtained using primers located at the 5′ or 3′ ends of the gene. These results were surprising given the fact that both the nuclear run-on assays and the promoter assays indicate that transcription of the *EPC-1* gene was relatively

constant. Alterations in the rate of transcription provide the simplest explanation for alterations in steady-state levels of hnRNA; however, this was clearly not the case for *EPC-1*. It is likely that the changes in steady-state levels of the hnRNA resulted from changes in hnRNA processing and/or hnRNA stability. This change in hnRNA abundance, combined with the apparent change in *EPC-1* mRNA stability, may account for the decrease in the steady-state levels of *EPC-1* mRNA after serum stimulation of quiescent fibroblasts.

DISCUSSION

The data presented here indicate that two mechanisms regulate cellular *EPC-1* mRNA levels. The first mechanism acts at the level of hnRNA abundance, and the second mechanism acts at the level of mRNA stability.

We have shown by extensive nuclear run-on analyses and promoter studies that transcription rates for the *EPC-1* gene do not change significantly under conditions in which mRNA levels change greatly (quiescent cells versus proliferating or senescent cells). Still, *EPC-1* hnRNA and mRNA levels show similar differences for each condition. Thus, the changes in hnRNA levels must result either from changes in processing rates or hnRNA stability. If an alteration in mRNA stability was the only mechanism controlling *EPC-1* mRNA levels, then hnRNA levels would not vary because transcription does not change significantly and the hnRNA would be processed at the same rate under all conditions. Thus, it seems that the rate of processing must be altered during the transition from a quiescent state to a proliferating state. It is possible that both splicing and stability of the *EPC-1* hnRNA are involved in the regulation of *EPC-1* mRNA levels in proliferating and senescent cells. The rate of splicing may be slowed, leading to retention of introns, which could in turn lead to destabilization of the *EPC-1* hnRNA. This occurs in both thymidine kinase and the *Xenopus* L1 gene regulation [Caffarelli et al., 1987; Gudas et al., 1988].

Regulation of gene expression at the hnRNA level has been observed in an increasing number of genes as the molecular tools become available to study differences in hnRNA levels. Examples of genes regulated at the hnRNA level include the proliferating cell nuclear antigen (PCNA), granulocyte-macrophage colony-stimulating factor, interleukin-2, glucose-6-phosphate dehydrogenase, and α-amidating monooxygenase [Chang et al., 1990, 1991; Akahane and Pluznik, 1992; Akahane and Pluznik, 1992; Umlauf et al., 1995; El Meskini et al., 1997; Stabile et al., 1998].

Expression of the PCNA gene is also decreased in senescent WI-38 fibroblast cells and is regulated by posttranscriptional mechanisms [Chang et al., 1990; Sell et al., 1992]. The PCNA hnRNA is present in senescent cells at levels comparable to that in proliferating cells. However, similar to *EPC-1*, PCNA mRNA levels are undetectable in senescent WI-38 fibroblasts [Chang et al., 1991] whereas the hnRNA is readily detected using RT-PCR. Under some conditions, the changes in PCNA hnRNA levels are regulated by changes in stability [Sell et al., 1992]. Both *EPC-1* and PCNA hnRNA are regulated during replicative senescence, and it is possible that a common mechanism may be involved. Thus, posttranscriptional regulation may occur during replicative senescence at multiple levels.

The *EPC-1* mRNA accumulates gradually and slowly over a matter of days as WI-38 fibroblasts enter quiescence. However, the decay of the mRNA levels is much more rapid on serum stimulation. The half-life of *EPC-1* mRNA in quiescent cells is 25–29 h. Disregarding transcription entirely, the *EPC-1* mRNA molecules already present have to be acted on to reduce their levels at the increased decay rate observed. The experimental data we have described is consistent with the presence of a serum-inducible labile protein that acts on the *EPC-1* mRNA to destabilize it. Several mRNA binding factors capable of destabilizing particular mRNA species have now been described [Ross, 1996]. It would be of interest to identify this protein and examine its expression level during replicative senescence. Increased expression might be responsible for a change in the stability of a subset of mRNA species within the senescent cell and thus might contribute to changes in gene expression, at the mRNA level, which occur during replicative senescence.

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